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LIPOLYTIC ACTIVITY OF *STAPHYLOCOCCUS ALBUS**

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ABSTRACT

Several indirect lines of evidence have converged to suggest that *C. acnes* and *S. albus* are sources of lipolytic enzymes in the pilosebaceous follicle and sebaceous duct, resulting in the liberation of free fatty acids, a process thought to be highly significant in the pathogenesis of acne. Direct experimental confirmation of the previously inferred role of *S. albus* has been obtained by incubation of known pure triglycerides with *S. albus*, and subsequent analysis of the resultant product with thin layer chromatography. In this *in vitro* system *S. albus* has been clearly demonstrated to have the capacity to cleave triglycerides resulting in the liberation of free fatty acids. These results are similar to those obtained in our previous studies with *C. acnes*.

In a previous paper (1) we reviewed the indirect evidence suggesting the probable role of *Corynebacterium acnes* as a source of lipolytic enzymes responsible for the formation of free fatty acids in human surface lipid by cleavage of fatty acids from the triglycerides of newly formed sebum (2–6). Direct experimental confirmation of this previously inferred role of *C. acnes* was then obtained by *in vitro* incubation of known pure triglycerides with *C. acnes* and subsequent analysis of the resultant product with thin layer chromatography. In this *in vitro* system *C. acnes* was clearly demonstrated to have the capacity to cleave triglycerides resulting in the liberation of free fatty acids.

The present study was undertaken as part of a systematic survey to determine whether other members of the cutaneous microflora possess lipolytic enzymes capable of hydrolyzing triglycerides to release free fatty acids. For this study, 5 short to long chain saturated triglycerides were chosen: tricaprylin, tricaprin, trilaurin, trimyristin, and tripalmitin, and 2 unsaturated triglycerides: tripalmitolein and triolein.

MATERIALS AND METHODS

1. Thin layer chromatography plates—silica gel, pre-coated, abrasion resistant, 20 x 20 cm from Brinkman Instrument, Inc. Westbury, New York.

2. 2′,7′-dichlorofluorescein from Eastman Organic Chemicals.

3. 2,2,4-trimethylpentane (iso-octane), b. 98.55–99.5° C. and iso-propyl acetate, b. 86.5–88.5° C. both from Matheson, Coleman, and Bell.

4. Chloroform and methanol-reagent grade from Merck.

5. (a) Triglycerides: tricaprylin, tricaprin, trilaurin, trimyristin, tripalmitin, tripalmitolein and triolein. (b) Diglycerides: dimyristin, dipalmitin, and diolein. (c) Monoglycerides: monomyristin, monopalmitin and monoolein. (d) Fatty acids: caprylic, capric, lauric, myristic, palmitic, palmitoleic, and oleic (all from Applied Science Laboratories, Inglewood, California). All lipid standards were prepared in glass distilled chloroform:methanol (2:1 v/v).

A basal medium was prepared for use in all growth studies. This consisted of 1% peptone (Bacto) and 0.5% yeast extract (Difco) in distilled water. Medium was distributed in 2 ml amounts into ether rinsed screw cap test tubes, and autoclaved at 15 lbs. pressure for 15 minutes.

All triglycerides were rechecked for purity in our laboratory by thin layer chromatography before further use. Tricaprylin, tripalmitolein, and triolein, all liquids at room temperature, were incorporated in the amount of 2 mg of triglyceride per 2 ml tube of room temperature medium. Those triglycerides which are solid at room temperature—tricaprin, trilaurin, trimyristin, and tripalmitin—were first suspended in acetone at room temperature, and then added dropwise to boiling medium to a total amount of 2 mg of triglyceride per 2 ml tube of medium.

Staphylococcus albus strains used in this study were obtained from comedones expressed from the nose or forehead of patients with acne vulgaris. Primary cultures were made anaerobically on Brain Heart Infusion Agar (Difco) supplemented with 1% dextrose. This medium and method of incubation is used routinely in our laboratory for the isolation of both *Staphylococci* and *Corynebacteria* from the skin. After gram staining, presumptive *S. albus* colonies were identified and subgrouped according to the Baird-Parker scheme (7). These gram positive cocci were coagulase negative, catalase positive, fermented dextrose

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anaerobically, fermented lactose, maltose, glycerol and mannitol aerobically, and were phosphate negative, and thus met the criteria of Baird-Parker for Staphylococcus sub-group VI. Cultures of *S. albus* grown in Brain Heart Infusion Broth (Difco) with constant shaking for 6 hours were used as inocula. Organisms were harvested and washed twice with sterile distilled water. The suspensions were standardized with distilled water to a final optical density of 80 in the Coleman Jr. spectrophotometer at 420 lambda. One tenth ml of *S. albus* suspension was inoculated into each tube. Controls of sterile basal media without triglycerides, sterile basal media with triglycerides, and inoculated basal media without triglycerides were included in each experiment. Tubes were incubated aerobically at 37° C. on a shaker for 24 hours.

Following incubation, the culture tubes were agitated to obtain even dispersion, and the contents transferred to glass stoppered extraction tubes. Five volumes of chloroform:methanol (2:1) were added to each extraction tube. After vigorous shaking for approximately 2 minutes, the tubes were centrifuged and the aqueous portion discarded. The residual organic solvent phase was transferred to a clean glass stoppered tube, and stored for approximately 4 hours at 15° C. We have noted that this step greatly facilitates removal of unwanted aqueous residues, and yields clean, clear solvent phases.

The solvent was then evaporated at room temperature in a stream of nitrogen to a volume of approximately 0.1 ml. This was streaked on TLC plates.

The one dimensional plates were developed first in iso-octane: isopropyl acetate (50:30) until the solvent front was approximately 2 cm from the top of the plate. The plate was then removed and air dried for 15 minutes. It was then rerun in the same solvent system to the same solvent front. This double development resulted in improved separation. The two dimensional plates were developed first in chloroform 95, methanol 5, and ammonia .08 until the solvent front moved within 4 cm of the top of the plate. The plate was then dried at room temperature for 15 minutes and turned 90° and developed in chloroform 8, acetone

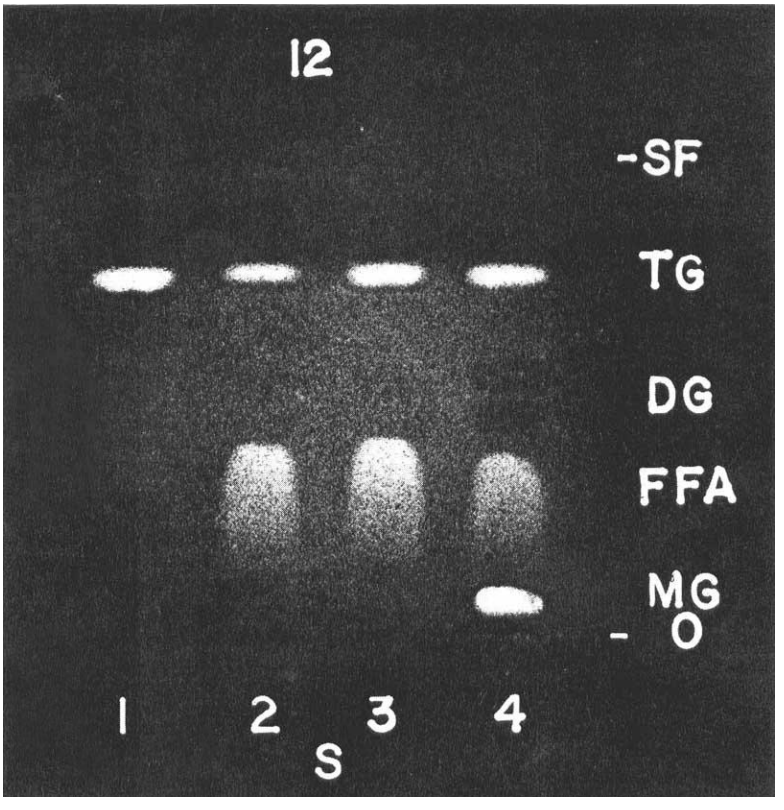

FIG. 1. TLC plate #12—Incubation of *S. albus* with trilaurin. O-origin, SF-solvent front. *Lane 1*—Basal medium plus trilaurin after incubation; *Lane 2*—Basal medium plus trilaurin plus *S. albus* (isolate S-1); after incubation; *Lane 3*—Basal medium plus trilaurin plus *S. albus* (isolate S-2) after incubation; *Lane 4*—Standard: trilaurin 100 mcg (TG), dimyristin 100 mcg (DG), lauric acid 100 mcg (FFA), monomyristin 100 mcg (MG), lecithin 100 mcg (O). (Doubly developed in iso-octane:iso-propyl acetate, (50:30).)

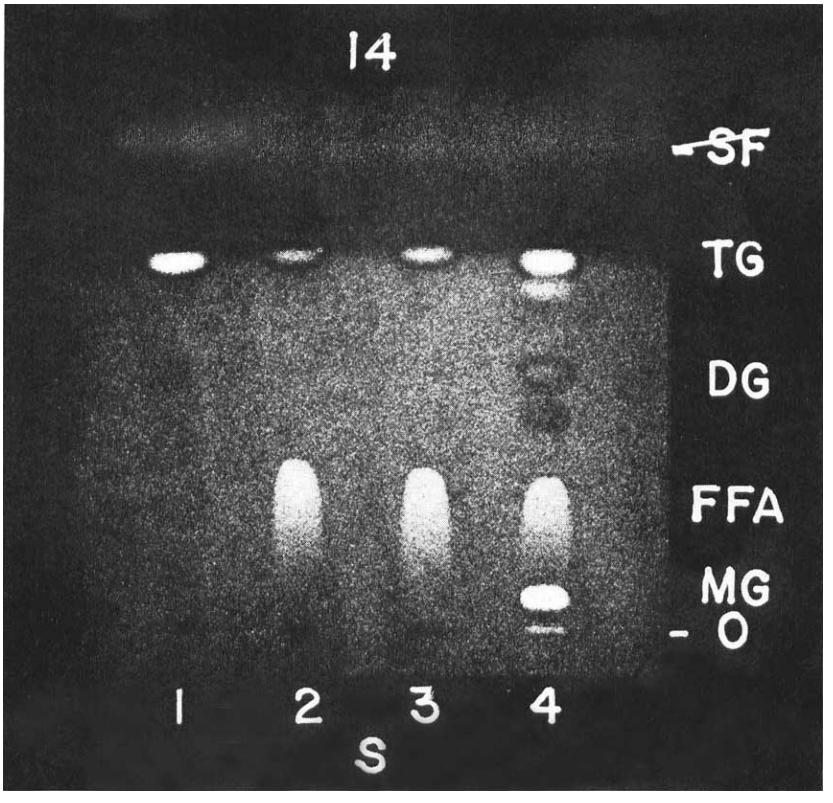

FIG. 2. TLC plate #14—Incubation of *S. albus* with trimyristin. O-origin SF-solvent front. *Lane 1*—Basal medium plus trimyristin after incubation; *Lane 2*—Basal medium plus trimyristin plus *S. albus* (isolate S-1) after incubation; *Lane 3*—Basal medium plus trimyristin, plus *S. albus* (isolate S-2) after incubation; *Lane 4*—Standard: trimyristin 100 mcg (TG), dimyristin 100 mcg (DG), myristic acid 100 mcg (FFA), monomyristin 100 mcg (MG), lecithin 100 mcg (O). An unidentified fluorescent spot appears just below the triglyceride standard in lane 4. (Doubly developed in iso-octane:iso-propyl acetate, (50:30).)

1.5, methanol 0.25, acetic acid 0.25, and water 0.8 until the solvent front was within 4 cm of the top of the plate.

In the one dimensional plates, one lane of each plate consists of known standard lipid components for identification. Additional lanes were run for extracts of media alone, extracts of individual triglycerides and media mixed, and extracts of bacteria and media mixed, to serve as controls.

Plates were then sprayed with 0.2% 2′,7′ dichlorofluorescein in ethanol for visualization of the lipids, and photographed in ultraviolet light in the Chromato-Vue made by Ultraviolet Light Products of San Gabriel, California, utilizing Kodalith Ortho film at f 5.6 for 10 seconds, and developed for 1 minute in Dektol.

RESULTS

Unless otherwise specifically noted, extracts cited in this section are chloroform:methanol (2:1) extracts, and all TLC plates are doubly developed in iso-octane:isopropyl acetate (50:30).

As noted previously (1) control plates demonstrated the absence of free fatty acids in extracts of sterile basal media and basal media inoculated with *S. albus*, thus serving as control observations for the experimental plates in which various triglycerides are incorporated in basal media inoculated with *S. albus*. A stationary, nonfluorescent spot is present at the point of origin in all lanes containing medium. Due to slight variations in photographic technique, it is clearly visible in plate XIV (Fig. 2), visible with difficulty in plate XII (Fig. 1) and not visible although present in plate XVI (Fig. 3). The lipid standards (lane 4 in all plates) move in ascending order as follows:

Phospholipids (O), monoglycerides (MG),

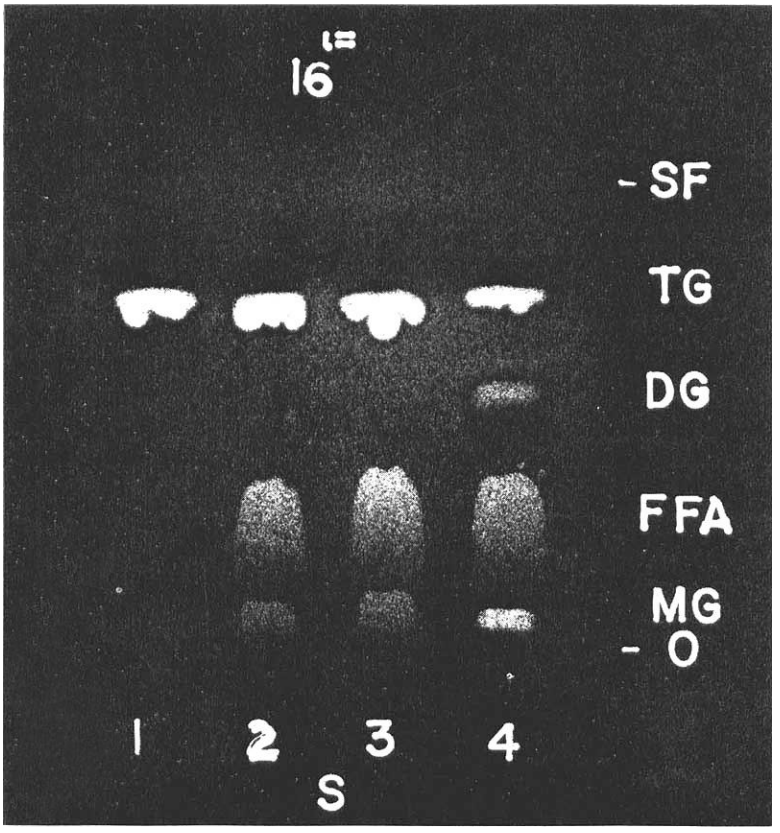

FIG. 3. TLC plate #16—Incubation of *S. albus* with tripalmitolein. O-origin, SF-solvent front. *Lane 1*—Basal medium plus tripalmitolein after incubation; *Lane 2*—Basal medium plus tripalmitolein plus *S. albus* (isolate S-1) after incubation; *Lane 3*—Basal medium plus tripalmitolein plus *S. albus* (isolate S-2) after incubation; *Lane 4*—Standard: tripalmitolein 100 mcg (TG), dipalmitin 100 mcg (DG), palmitoleic acid 100 mcg (FFA), monopalmitin 100 mcg (MG), lecithin 100 mcg (O). (Doubly developed in iso-octane:isopropyl acetate, (50:30).)

free fatty acid (FFA), diglycerides (DG), with the characteristic double spot representing the 1,3 and 1,2 structures, and triglycerides (TG).

TLC plate XII shown in Fig. 1 demonstrates the lipolytic activity of *S. albus* acting on a saturated triglyceride, trilaurin, to release a free fatty acid.

TLC plate XIV shown in Figure 2 is another representative plate demonstrating the capacity of *S. albus* to hydrolyze a longer straight chain saturated triglyceride trimyristin, to release free fatty acids.

TLC plate XVI shown in Figure 3 demonstrates the lipolytic capacity of *S. albus* in liberating free fatty acid from a longer straight chain unsaturated triglyceride, tripalmitolein. The presence of a fluorescent spot in lane 2 and 3 migrating at RF of the monoglyceride standard suggests the possibility that in this experiment, the lipolytic enzymes available from *S. albus* were insufficient to completely hydrolyze all fatty acid molecules from the glycerol skeleton, thus leaving some molecules from which only 2 free fatty acids had been cleaved resulting in the presence of a monoglyceride.

The leading edge of the FFA spot in lanes 2 and 3 of plate XII, lanes 2 and 3 of plate XIV, and lane 3 of plate XVI migrate beyond the FFA standard. Our TLC studies with varying concentrations of single free fatty acids and with mixtures of FFA demonstrate that these differences in RF in this system can be a function of either concentration or composition. Figure 4 demonstrates the effect of concentration on RF. Myristic acid was streaked at the origin in concentrations varying from 10 to 1000 mcg

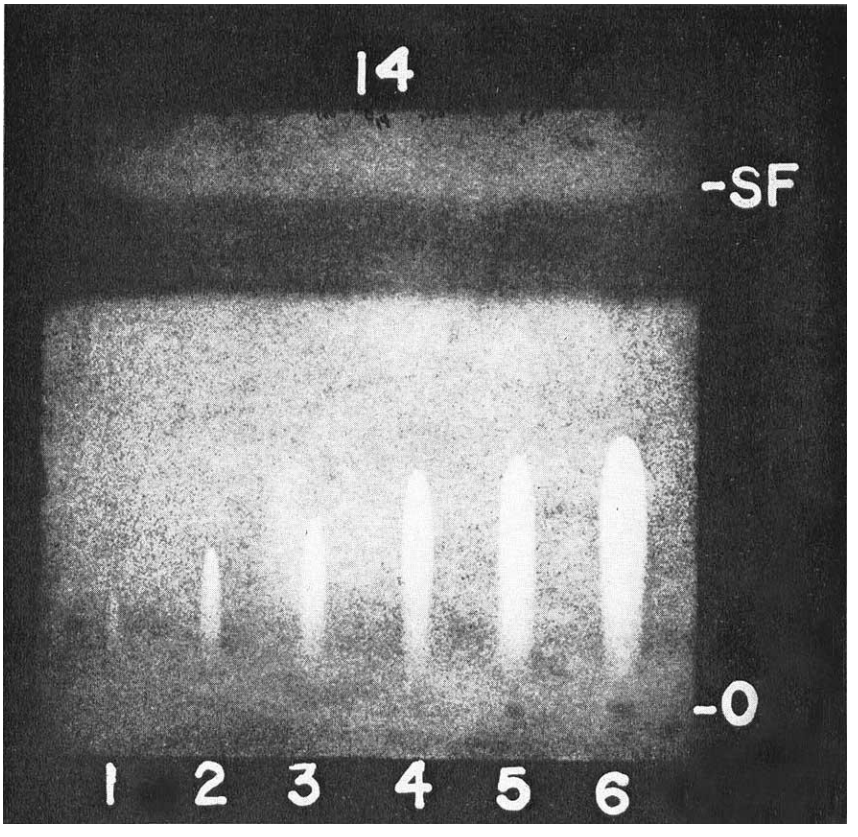

FIG. 4. Effect of concentration on RF—Myristic acid. O-origin, SF-solvent front. *Lane 1*—Myristic acid 10 mcg; *Lane 2*—Myristic acid 50 mcg; *Lane 3*—Myristic acid 100 mcg; *Lane 4*—Myristic acid 250 mcg; *Lane 5*—Myristic acid 500 mcg; *Lane 6*—Myristic acid 1000 mcg. (Doubly developed in iso-octane:iso-propyl acetate, (50:30).)

and the resultant increasing RF values can be clearly seen. Figure 5 demonstrates the effect of composition on RF. A series of free fatty acids of ascending molecular weight was streaked at the origin in lanes 1 through 5 in equal amounts of 100 mcg each. Lane 6 represents 20 mcg each of the FFA in lanes 1 through 5, a total of 100 mcg. Clearcut differences in migration are visible. Whether the alterations in RF value observed (Figs. 1–3) represent differences in composition of the various FFA derived from lipolysis of triglycerides is being subjected to further study, since degradation to form FFA of shorter chain lengths is potentially significant in the pathogenesis of acne.

Utilizing the 2 dimensional method developed by Dr. George Rouser as described in *Methods* above, spots corresponding to the FFA standard were scraped off a 1 dimensional TLC plate, extracted with chloroform:methanol (2:1), and the identity of the FFA confirmed by 2 dimensional chromatography.

In addition to the representative TLC plates shown in Figures 1, 2, and 3 in which the effect of the incubation of *S. albus* with trilaurin, trimyristin, and tripalmitolein in releasing free fatty acids from triglycerides was demonstrated, similar experiments with tricaprylin, tricaprin, tripalmitin, and triolein, were carried out with similar results. In each case free fatty acids were clearly shown to be liberated from the pure triglyceride after incubation with *S. albus*.

DISCUSSION

This preliminary study has clearly demonstrated the capacity of *S. albus* (Staphylococcus sub-group VI-Baird-Parker) to cleave *in vitro* a series of triglycerides resulting in the liberation of free fatty acids. The triglycerides chosen in this initial study were 5 short to long chain

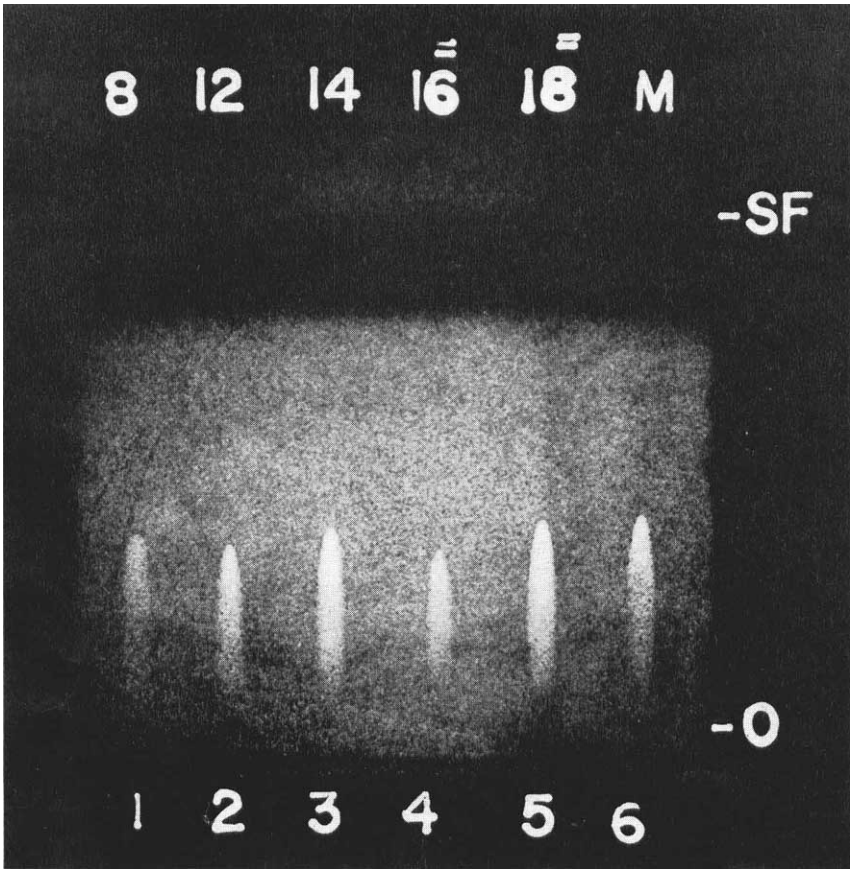

FIG. 5. Effect of composition on RF. O-origin, SF-solvent front. *Lane 1*—Caprylic acid 100 mcg; *Lane 2*—Lauric acid 100 mcg; *Lane 3*—Myristic acid 100 mcg; *Lane 4*—Palmitoleic acid 100 mcg; *Lane 5*—Oleic acid 100 mcg; *Lane 6*—A mixture of 20 mcg of each of the 5 above FFA. (Doubly developed in iso-octane:iso-propyl acetate, (50:30).)

saturated triglycerides and 2 straight chain unsaturated triglycerides. These findings were in the process of being written up when an independent report by Dr. Ruth Freinkel (9) was published also describing the lipolytic activity of *Staphylococcus albus*. The lipids used in that study were olive oil, which is composed of mixed glycerides of oleic acid, 83.5%, of palmitic acid, 9.4%, of linoleic acid, 4%, of stearic acid, 2%, of arachidic acid, 0.9%, and squalene, up to 0.7%, phytosterol and tocopherols about 0.2% and an unidentified lipid used in a spirit blue indicator system (Difco). Further study will be necessary to clarify the exact spectrum of lipolytic activity of *S. albus* and identify any variations in glycerides attacked as well as any preferential order of degradation.

The studies reported in this paper are with 2 isolates of *S. albus* isolated from patients with acne. Although no obvious differences in lipolytic activity were observed *in vitro* under the conditions of this experiment, the possibility still remains that various strains may possess differing lipolytic activity, and might thus possibly have different degrees of pathogenicity in terms of acneigenesis. Further, the precise patterns of lipolytic activity of individual strains may vary, resulting in the production of different patterns of composition of free fatty acids. In view of the probable differing degrees of irritancy of various free fatty acids, this may be a significant factor. Further studies utilizing gas chromatography to analyze in detail the free fatty acids identified by TLC are now under way.

These experiments coupled with the previously reported ones (1) clearly demonstrate the lipo-

lytic activity of *S. albus* and *C. acnes*. Additional studies are needed to determine the relative significance of these sources of lipolytic enzymes in the sebaceous duct and the pilosebaceous follicle as opposed to those derived from other organisms present including *Pityrosporum* species (10), *Demodex folliculorum*, and other bacteria, particularly the lipophilic diphtheroids, as well as endogenous lipolytic enzymes if any. Studies to evaluate the role of these organisms are now under way, and initial results indicate that both *Pityrosporum ovale* (10) and the lipophilic diptheroids (11) possess similar lipolytic enzymes.

However, as noted previously (1) there is evidence suggesting that both *C. acnes* and *S. albus* are important sources of lipolytic activity in the sebaceous duct and the pilosebaceous follicle. The administration of tetracycline systemically results in a striking reduction in the concentration of free fatty acids in sebum, which can be correlated with improvement of clinical acne (5). This reduction in the concentration of free fatty acids is well established by the end of the 3rd week of tetracycline therapy. Upon discontinuation of therapy there is a trend toward resumption of pre-treatment levels of free fatty acids. This trend is evident during the first 3 week period after cessation of treatment. These observations correlate well with the 2 weeks required for the marked drop in the density of *C. acnes* and *S. albus* observed after the initiation of tetracycline therapy and with the 2 weeks required for the return to normal density after the discontinuation of tetracycline therapy (12). These observations coupled with the evidence for the lipolytic activity for *C. acnes* and *S. albus*, would suggest that these organisms may be an important source of lipolytic activity in the *in vivo* situation. However, other sources such as *Pityrosporum ovale* are also likely to be present to account for the fact that tetracycline therapy results in only about a 50% reduction in sebum FFA (5, 10).

SUMMARY

Staphylococcus albus has been demonstrated to have the capacity to cleave triglycerides *in vitro*, resulting in the liberation of free fatty acids. This, coupled with previous evidence regarding the role of *C. acnes* gives direct experimental confirmation to the role of these 2 organisms as sources of lipolytic activity of the sebaceous duct and pilosebaceous follicle. Study is now under way to evaluate the role and relative significance of other cutaneous microflora as sources of lipolytic activity.

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REFERENCES

1. Reisner, R. M., Silver, D. Z., Puhvel, M. and Sternberg, T. H.: Lipolytic activity of *Corynebacterium acnes*. J. Invest. Derm., *51:* 190–198, 1968.
2. Kellum, R. E.: Human sebaceous gland lipids. Arch. Derm., *95:* 218, 1967.
3. Nocolaides, N. and Wells, G. C.: On the biogenesis of the free fatty acids in human skin surface fat. J. Invest. Derm., *29:* 423, 1957.
4. Scheimann, L. G., Knox, J., Sher, D. and Rothman, S.: Role of bacteria in formation of free fatty acids on human skin surface. J. Invest. Derm., *34:* 171, 1960.
5. Freinkel, R. K., Strauss, J. S., Yip, S. Y. and Pochi, P. E.: Effect of oral tetracycline on fatty acids of human sebum. New Eng. J. Med., *273:* 850, 1965.
6. Pochi, P. E. and Strauss, J. S.: Antibiotic sensitivity of *Corynebacterium acnes* (Propionibacterium acnes). J. Invest. Derm., *36:* 423, 1961.
7. Baird-Parker, A. C.: The classification of staphylococci and micrococci from worldwide sources. J. Gen. Microbiol., *38:* 363, 1965.
8. Rouser, G.: Personal Communication.
9. Freinkel, R. K.: The origin of free fatty acids in sebum I. Role of coagulase negative staphylococci. J. Invest. Derm., *50:* 186, 1968.
10. Reisner, R. M.: *Pityrosporum ovale* and tetracycline—resistant acne. Presented before the American College of Physicians, Palm Spring, California, February 23–25, 1968.
11. Reisner, R. M. and Puhvel, M.: Unpublished data.
12. Goltz, R. W. and Saemunder, K.: Oral tetracycline treatment on bacterial flora in acne vulgaris. Arch. Derm., *93:* 92, 1966.